Biochimica et Biophysica Acta 897 (1987) 52–56
Elsevier

BBA 73188

Thiazides stimulate calcium absorption in urinary bladder of winter flounder

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(Received 23 June 1986)

Key words: Hydrochlorothiazide; Sodium/chloride cotransport; Sodium–calcium exchange; Ouabain; Distal convoluted tubule

Thiazides inhibit voltage-independent NaCl absorption in the urinary bladder of the winter flounder presumably by blocking an electroneutral mucosal Na/Cl co-transporter. As thiazides stimulate calcium absorption in mammalian distal convoluted tubule while inhibiting NaCl absorption, we studied the effects of hydrochlorothiazide (HCTZ) on unidirectional ^{45}Ca fluxes and intracellular electrical potential in short-circuited bladders to examine possible mechanisms of HCTZ effects on calcium transport. Basal secretory calcium flux was, on average, slightly larger than absorptive flux, reflecting small net calcium secretion. Mucosal addition of HCTZ (10^{-4} M) stimulated absorptive calcium flux by 46% while the secretory flux was unaltered. Thus, HCTZ tended to induce net calcium absorption. Pre-treatment with serosal ouabain (10^{-4} M) attenuated the HCTZ-induced increase in absorptive calcium flux. Moreover, HCTZ hyperpolarized the mucosal membrane potential by 18% as measured by conventional open-tip microelectrodes. These effects of HCTZ are consistent with the hypothesis that HCTZ indirectly stimulates Na/Ca exchange located at the serosal membrane. In conclusion, HCTZ in flounder urinary bladder, as in mammalian distal convoluted tubule, simultaneously inhibits NaCl absorption and stimulates calcium absorption. This study expands on the functional similarities between the flounder urinary bladder and the mammalian distal convoluted tubule.

Introduction

The urinary bladder of higher teleosts contributes significantly to salt and water homeostasis in a manner which is complimentary to the function of the kidney [1]. In fact, the urinary bladder of such animals (e.g., *Pseudopleuronectes americanus,* or winter flounder) is an extension of the mesonephric duct. Thus, unlike the endodermal origin of amphibian urinary bladder, the urinary bladder of higher teleosts is anatomically and functionally an extension of the kidney [1,2] and may represent a suitable transport model of some segment of the distal nephron.

The flounder urinary bladder is a high-resistance epithelium that actively absorbs sodium chloride in a voltage-independent fashion [2,3]. The rates for Na^+ and Cl^- net absorption are virtually equal and exhibit interdependence of each ion on the other [2,3]. Moreover, sodium chloride transport is not dependent on the presence of potassium in the mucosal fluid. Recently, Stokes demonstrated that thiazide diuretics inhibit net sodium chloride absorption presumably by blocking an electroneutral Na/Cl co-transporter located

Abbreviations: HCTZ, hydrochlorothiazide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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at the mucosal membrane [3]. Thus, the flounder urinary bladder may prove to be a unique model of sodium chloride absorption and may share several functional similarities with the mammalian distal convoluted tubule.

Since thiazides stimulate calcium absorption in the early segment of mammalian distal confoluted tubule while simultaneously inhibiting net sodium chloride absorption [4], we examined in the current study the effects of hydrochlorothiazide (HCTZ) on unidirectional ^{45}Ca fluxes and on intracellular electrical potential in the short-circuited flounder urinary bladder to gain some insights into the action of thiazides on calcium transport.

Methods

The methods used are, with slight modifications, those previously described [3,5]. Urinary bladders were dissected from flounders and mounted as flat sheets in modified Ussing chambers. Exposed bladders (area = 0.80 cm^2) were continuously short-circuited and were bathed on both sides with a solution containing in mM: Na^+, 147.5; Cl^-, 147.5; K^+, 2.5; Ca^{2+}, 1.5; Mg^{2+}, 1.0; Hepes, 15.0; glucose, 5.0. The mucosal (M) and serosal (S) chambers were continuously bubbled with 99% O_2/1% CO_2 and the pH was approximately 7.5. Verapamil (10 μM) was added to the fluid which bathed the bladders after dissection and before mounting. This was necessary in order to reduce vigorous contraction of bladder smoth muscle [3,5]. After mounting, however, the verapamil was washed off and fresh bathing fluid without verapamil was added to the mucosal and serosal chambers (10 ml each). Whether transient application of verapamil reduces transepithelial calcium fluxes in this tissue remains undetermined. It has been previously shown, however, that verapamil does not have any effect on monovalent ion fluxes in this tissue [3].

Transepithelial ^{45}Ca fluxes were measured as previously detailed for frog skin [6]. One hundred μCi of ^{45}Ca in 100 μl were added either to the serosal or mucosal solution and the rate of appearance of the isotope was measured on the opposite side. Samples of 50 μl each were taken from both chambers every 15 or 30 min, added to scintillation vials, each containing 9.5 ml Aquasol II (New England Nuclear) and 0.5 ml glacial acetic acid, and the radioactivity was counted by liquid scintillation spectrometry. In preliminary studies, it was determined that unidirectional calcium fluxes reach a steady state within 90 min. Thus, we allowed at least 90 min for isotope equilibration, followed by two or three 30-min control collections. Hydrochlorothiazide (Sigma) was then added to the mucosal bath and three 15-min collections were taken.

In all experiments, the short-circuit current (I_{sc}) was measured by continuously calmping the transepithelial voltage to 0 mV. To assess the transepithelial resistance (R_T), the transepithelial voltage was clamped at 10 mV for a few seconds every 5–10 min. The resultant deflection in transepithelial current was used to calculate R_T as previously described [6]. Bladders exhibiting basal R_T below 250 $\Omega \cdot cm^2$ were discarded.

In a separate group of bladders, potential-sensing microelectrodes were used to monitor the intracellular electrical potential as previously described [7]. In this set of experiments, bladders were mounted horizontally, mucosal surface upwards, and were continuously short-circuited. Cells were impaled across the mucosal cell membrane using conventional open-tip micropipettes filled with 0.5 M KCl [7]. The potential difference was measured with reference to the mucosal medium. The outer tip diameter of the micropipette was less than 0.2 μm and displayed resistances between 60 and 100 MΩ when tested in the bathing solution.

All results are expressed as means $\pm$ S.E. for the group. Data from average values of control periods were compared with those of experimental periods of the same bladders using a paired t test. Comparison between groups of bladders matched by R_T was done with an unpaired t test. $P > 0.05$ was considered not significant (n.s.).

Results

The mucosa-to-serosa calcium flux (JCa^{MS}), i.e., absorptive flux, exhibited an average value of 104 ± 20 pmol/cm^2 per h ($n = 20$ bladders) in the basal state. The average R_T in these bladders was 724 ± 83 $\Omega \cdot cm^2$. In another group of 12 bladders

exhibiting a comparable R_T of 592 ± 57 $\Omega \cdot cm^2$ (P = n.s.), the serosa-to-mucosa calcium flux (JCa^{SM}), i.e. ,secretory flux, was sligthly larger (180 ± 40 pmol/cm^2 per h, $P = 0.05$). Thus, in the absence of an electrochemical gradient for calcium, the flounder bladder tends to express a small degree of net calcium secretion averaging 76 pmol/cm^2 per h.

Fig. 1 depicts the effects of mucosal HCTZ (10^{-4} M) on JCa^{MS} and R_T. In each bladder studied, HCTZ increased JCa^{MS}, on average by 46% (from 137 ± 30 to 200 ± 40 pmol/cm^2 per h, $n = 9$, $P < 0.005$). As previously reported [3], HCTZ also decreased R_T, on average by 14% (from 630 ± 101 to 541 ± 87 $\Omega \cdot cm^2$, $P < 0.005$). Fig. 2 shows the effects of mucosal HCTZ (10^{-4} M) on JCa^{SM} and R_T in a separate group of 10 bladders. Here, HCTZ had no effect on JCa^{SM} (161 ± 40 vs. 160 ± 50 pmol/cm^2 per h in control period, P = n.s.), while R_T was uniformly decreased, on average by 18% (from 597 ± 64 to 491 ± 62 $\Omega \cdot cm^2$, $n = 10$, $P < 0.001$) similar to the reduction in R_T shown in Fig. 1. Thus, HCTZ, by preferentially stimulating JCa^{MS}, converted the average basal net secretion of calcium (23 pmol/cm^2 per h) to net absorption (30 pmol/cm^2 per h).

In an additional group of three bladders, we examined the effect of pre-treatment with serosal ouabain (10^{-4} M) on the response to mucosal HCTZ (Table I). Ouabain was added at the beginning of the isotope equilibration period (90 min).

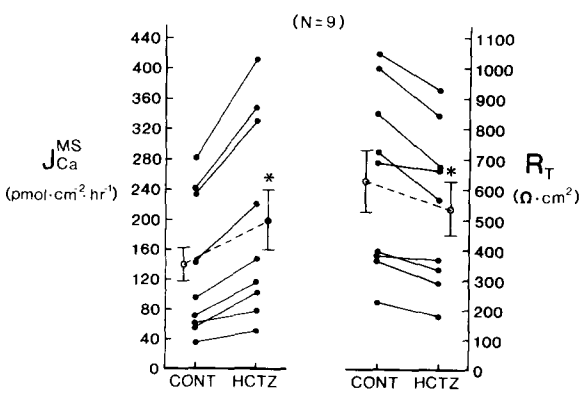

Fig. 1. The effect of mucosal hydrochlorothiazide (HCTZ), 10^{-4} M, on the absorptive calcium flux (JCa^{MS}) and on transepithelial resistance (R_T) in nine bladders. * $P < 0.005$ compared to control period (CONT).

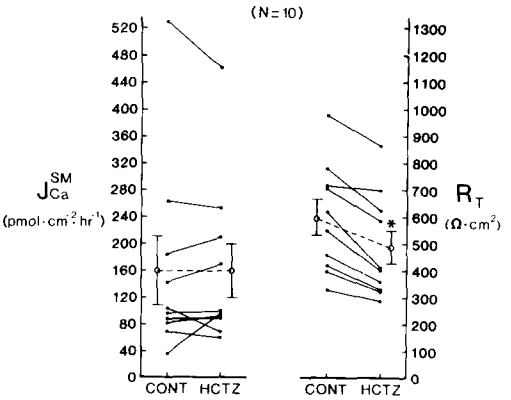

Fig. 2. The effect of mucosal hydrochlorothiazide (HCTZ), 10^{-4} M, on the secretory calcium flux (JCa^{SM}) and on transepithelial resistance (R_T) in 10 bladders. * $P < 0.001$ compared to control period (CONT).

JCa^{MS} and R_T were determined in a 60 min control period, then in a 45 min experimental period beginning with the addition of mucosal HCTZ (10^{-4} M). As shown in Table I, pretreatment with ouabain prevented the increase in JCa^{MS} as well as the fall in R_T induced by HCTZ addition.

Fig. 3 depicts the effects of mucosal HCTZ (10^{-4} M) on I_{sc} and on the mucosal membrane potential (ψ^{MC}). In each of five bladders studied, HCTZ hyperpolarized ψ^{MC} by an average of 10.7 ± 2.5 mV ($P < 0.02$ compared to virtually no change in stable time control values) from -58.4

TABLE I

EFFECT OF OUABAIN PRE-TREATMENT ON THE RESPONSE TO HYDROCHLOROTHIAZIDE

Ouabain (10^{-4} M) was added to the serosal bath at the beginning of the isotope equilibration period followed by determination of the absorptive calcium flux (JCa^{MS}) and transepithelial resistance (R_T) in the control period (Period I) and following the addition of hydrochlorothiazide (HCTZ), 10^{-4} M, to the mucosal bath (Period II). The short-circuit current was virtually zero in both periods ($n = 3$ bladders).

	Period I (ouabain)		Period II (ouabain + HCTZ)
JCa^{MS} (pmol/cm^2 per h)	84 ± 14	(P = n.s.)	98 ± 6
R_T ($\Omega \cdot cm^2$)	667 ± 109	(P = n.s.)	647 ± 94

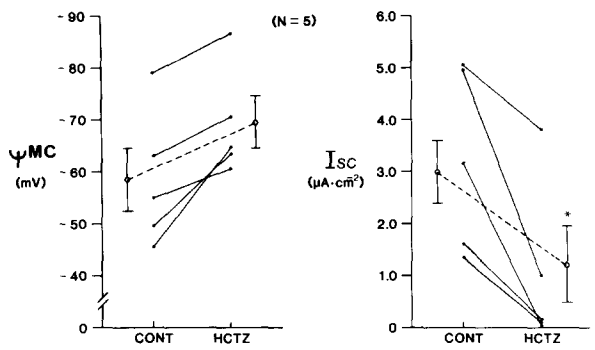

Fig. 3. The effect of mucosal hydrochlorothiazide (HCTZ), 10^{-4} M, on short-circuit current (I_{sc}) and on the intracellular electrical potential (ψ^{MC}) measured by impaling bladder cells across the mucosal cell membrane. * $P < 0.05$ compared to control period (CONT). In the open-circuited state, the recorded transepithelial voltate was mucosa-positive with respect to the serosal bath.

± 13 mV in the basal state, to -69.0 ± 10 mV after HCTZ addition. Concomitantly, HCTZ reduced I_{sc} from 3.0 ± 1.5 to 1.2 ± 1.5 $\mu A/cm^2$ ($n = 5$, $P < 0.05$). In the urinary bladder of the winter flounder, I_{sc} is totally accounted for by electrogenic potassium secretion [5]. This effect is responsible for the mucosa-positive transepithelial voltage which is recorded in the open-circuited state [5]. The HCTZ-induced reduction of I_{sc} is similar to that seen in previously reported studies [3,8].

Discussion

The results of the current study demonstrate that mucosal treatment by HCTZ, in the absence of an electrochemical gradient for calcium, preferentially stimulates the absorptive calcium flux in the urinary bladder of the winter flounder without altering the secretory flux. Thus, HCTZ reverses the tendency for basal net calcium secretion into net calcium absorption. Moreover, HCTZ hyperpolarizes the mucosal membrane potential. The effects of HCTZ in decreasing short-circuit current and transepithelial resistance confirm previously reported studies [3,8].

The stimulation by HCTZ of the absorptive calcium flux is most likely accounted for by an increase in the rate of transport through a cellular rather than a paracellular (shunt) pathway. This is supported by the absence of any increase in the opposite, or secretory, calcium flux with HCTZ administration, despite a fall in transepithelial resistance. It is interesting to note that Stokes has concluded that the reduction in transepithelial resistance induced by HCTZ could be totally accounted for by a decrease in the cellular rather than the paracellular component of total tissue resistance [8]. In our studies, the abrogation of the effect of HCTZ on transepithelial resistance by ouabain pre-treatment further supports this conclusion.

The mechanism underlying the HCTZ-induced increase in the absorptive calcium flux remains to be elucidated. Theoretically, it may involve stimulation of calcium exit from cells to the serosal medium by one or both of two processes: $(Ca^{2+} + Mg^{2+})$-ATPase or Na/Ca exchanger located at the serosal membrane. Direct evidence for the involvement of either process in the flounder urinary bladder is currently lacking. However, the data from the current study are consistent with a role for a serosal Na/Ca exchanger powered by the serosal-to-cell Na^+ gradient established by the activity of serosally located $(Na^+ + K^+)$-ATPase. This gradient, when increased, would result in enhanced calcium exit from the cells to the serosal medium. A steeper electrochemical gradient would be expected with hyperpolarization of the intracellular electrical potential and/or a decrease in cytosolic Na^+ activity as might be anticipated when mucosal NaCl entry is blocked by thiazides. Conversely, the Na^+ gradient can be greatly reduced if cell Na^+ activity is increased and/or if the intracellular electrical potential depolarizes as would be expected with inhibition of $(Na^+ + K^+)$-ATPase by ouabain pre-treatment, resulting in a marked inhibition of calcium exit from the cell via the putative serosal Na/Ca exchanger. Thus, the results of the current study are consistent with a role for a putative serosal Na/Ca exchange process in mediating the effects of HCTZ. Further direct evidence may be required to confirm this hypothesis. For instance, it may be necessary to show that serosal Na^+ substitution results in inhibition of thiazide-stimulated calcium absorption, and that Na/Ca exchange can be demonstrated in serosal-membrane vesicle preparations.

It is noteworthy that the magnitude of the

unidirectional calcium fluxes reported in this study are relatively small, perhaps suggesting a low intrinsic permeability to calcium in the flounder urinary bladder. However, these calcium fluxes are of relatively comparable magnitude to those measured in similar high-resistance epithelia, such as the toad urinary bladder [9] or the isolated frog skin epithelium in the basal state [6]. On the other hand, it appears that the net calcium absorption is larger in magnitude in the mammalian distal convoluted tubule. For instance, in the isolated perfused rabbit distal convoluted tubule, net calcium reabsorption averaged 0.21 pmol/mm per min [10]. When expressed in terms of tubule surface area, this value appears much larger than flux measurements obtained in the flounder urinary bladder.

In summary, this study expands on some of the functional similarities of transport properties exhibited by the urinary bladder of the winter flounder and the early segment of mammalian distal convoluted tubule. In both tissues, thiazide diuretics such as hydrochlorothiazide inhibit NaCl absorption while simultaneously stimulating calcium absorption. Despite the widespread use of thiazides, there is surprisingly very little information on their mechanism of action in electrolyte transport. The use of the flounder urinary bladder may prove very helpful in elucidating the cellular mechanisms of the action of thiazides on monovalent and divalent ion transport. The results of the current study are compatible with the hypothesis that the action of hydrochlorothiazide on calcium transport may be mediated by stimulation of serosal Na/Ca exchange. Further studies are required, however, to confirm this postulate.

Acknowledgments

This work was supported by National Institutes of Health Research Grant R01-AM-33138 and Training Grant T32-AM-07006. F.N.Z. is a recipient of a fellowship from the National Kidney Foundation and from the Measey Foundation. E.K. is a recipient of a Clinician Scientist Award from the American Heart Association. This work was presented in part at the National Meeting of the American Federation for Clinical Research, Washington, DC, 1986, and appeared in Abstract form (Clin. Res. 34 (1986) 703A).

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